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Comparison of Subtyping Methods for Differentiating *Salmonella enterica* Serovar Typhimurium Isolates Obtained from Food Animal Sources

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Molecular characterization (e.g., DNA-based typing methods) of *Salmonella* isolates is frequently employed to compare and distinguish clinical isolates recovered from animals and from patients with food-borne disease and nosocomial infections. In this study, we compared the abilities of different phenotyping and genotyping methods to distinguish isolates of *Salmonella enterica* serovar Typhimurium from different food animal sources. One hundred twenty-eight *S. enterica* serovar Typhimurium strains isolated from cattle, pigs, chickens, and turkeys or derived food products were characterized using pulsed-field gel electrophoresis (PFGE), repetitive element PCR (Rep-PCR), multilocus sequence typing (MLST), plasmid profiling, and antimicrobial susceptibility testing. Among the 128 *Salmonella* isolates tested, we observed 84 Rep-PCR profiles, 86 PFGE patterns, 89 MLST patterns, 36 plasmid profiles, and 38 susceptibility profiles. The molecular typing methods, i.e., PFGE, MLST, and Rep-PCR, demonstrated the best discriminatory power among *Salmonella* isolates. However, no apparent correlation was evident between the results of one molecular typing method and those of the others, suggesting that a combination of multiple methods is needed to differentiate *S. enterica* serovar Typhimurium isolates that genetically cluster according to one particular typing method.

Nontyphoidal salmonellae are among the foremost bacterial pathogens implicated in food-borne gastroenteritis worldwide. For the United States, a 1999 report estimated that there are approximately 1.4 million cases of nontyphoidal *Salmonella* infections annually, resulting in 17,000 hospitalizations and 585 deaths annually (15). In developed countries, *Salmonella* is most often transmitted to humans through the food chain, with over 95% of salmonellosis cases attributable to the consumption of undercooked or mishandled beef, chicken, eggs, turkey, or pork (1, 26). In the United States, *Salmonella* is responsible for an estimated 26% of all infections caused by bacterial food-borne pathogens (15), with the annual economic cost of lost wages, increased medical care, and loss of life estimated to be between 2.3 and 3.6 billion dollars each year (5, 11).

Among the over 2,400 serovars identified within *Salmonella enterica* subsp. *enterica*, *S. enterica* serovar Typhimurium continues to be one of the most frequently recovered from food animals worldwide (10, 21, 30). In the United States, it is among the top four most frequently detected *Salmonella* serotypes from diagnostic isolates recovered from cattle, swine, chickens, and turkeys (10). Because of its broad host range, *S. enterica* serovar Typhimurium is also one of the most common serotypes isolated from human clinical cases of food-borne salmonellosis (6). Additionally, serovar Typhimurium isolates often exhibit multidrug-resistant phenotypes (28, 30). As a result, monitoring antimicrobial susceptibility profiles as well as molecular genetic types is critical for characterizing outbreaks and guiding anti-infective therapy when warranted.

Knowledge of how *Salmonella* disseminates through the food chain is important in understanding how food animals and food-processing procedures contribute to food contamination and subsequent human infection. In addition, more informative typing methods may help to estimate the numbers of human cases attributable to certain animal sources. Currently, pulsed-field gel electrophoresis (PFGE) is the standard typing method for *Salmonella* outbreak investigations (24) and is suitable for examining epidemiologically related strains. However, because PFGE analysis of *S. enterica* serovar Typhimurium strains indicates weak clonality within the isolate population, tracing back sporadic cases is more difficult. In an attempt to better estimate the numbers of human cases attributable to certain animal sources, we evaluated and compared a number of genotypic and phenotypic typing methods, including repetitive element PCR (Rep-PCR), PFGE, multilocus sequence typing (MLST), antimicrobial susceptibility profiling, and plasmid profiling, for the ability to distinguish among a collection of *S. enterica* serovar Typhimurium isolates collected from different food animal origins.

MATERIALS AND METHODS

Bacterial strains. One hundred twenty-eight *Salmonella enterica* serovar Typhimurium strains recovered from food animal diagnostic samples ($n = 74$) or derived food products collected at slaughter and processing ($n = 54$) were used in this study. Among the 128 isolates studied, 32 strains representing each of the major food animal sources were randomly selected from the FDA Center for Veterinary Medicine's culture collection, which includes isolates from the National Antimicrobial Resistance Monitoring System—Enteric Bacteria (30). The representative isolates originated from cattle (including 12 from ground beef

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samples), swine (including 13 from ground pork samples), turkeys (including 16 from ground turkey samples), and chickens (including 13 from ground chicken and chicken carcasses). Isolates were serotyped by the National Veterinary Services Laboratory (Ames, IA) or with commercial Difco antisera (Becton Dickinson and Company, Sparks, MD) according to the manufacturer's instructions. Isolates were grown on Trypticase soy agar plates supplemented with 5% defibrinated sheep blood (Becton Dickinson Microbiology Systems, Sparks, MD) and stored in Trypticase soy broth (Becton Dickinson) containing 15% glycerol at −80°C until needed.

Antimicrobial susceptibility testing. Antimicrobial MICs were determined using the Sensititre semiautomated antimicrobial susceptibility system (Trek Diagnostic Systems, Cleveland, OH) per the manufacturer's instructions and were interpreted according to the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) standards for broth microdilution methods (18). The following antimicrobials were tested: amoxicillin-clavulanic acid, ampicillin (Amp), apramycin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin (Str), sulfamethoxazole (Sul), tetracycline, and trimethoprim-sulfamethoxazole. *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms.

Plasmid profiling. Isolates were cultured overnight in Luria-Bertani broth, and bacterial pellets were harvested by centrifugation at 15,000 × *g*. Isolation of plasmid DNA was accomplished by using a Millipore plasmid purification kit (Millipore, Bedford, MA). The plasmid DNAs were separated electrophoretically in 13-cm by 16-cm gels made of 0.7% agarose in 1× Tris-borate-EDTA (TBE) buffer at room temperature for 90 min at 100 V. The gels were stained with ethidium bromide to visualize the plasmids. The visualized bands were normalized and sized by comparing the plasmids' relative migration to that of a supercoiled ladder (Invitrogen, Carlsbad, CA). To test reproducibility, plasmid DNAs were also isolated using a second method (Wizard Plus SV minipreps; Promega, Madison, WI) and analyzed as described above to ensure that the original profiles were representative of the plasmid contents of the strains.

Rep-PCR. Template DNA was prepared by guanidinium thiocyanate isolation as described by Pitcher et al. (20). Rep-PCRs were carried out using a commercially available typing kit (Bacterial Barcodes, Houston, TX) with the provided Uprime-RI primer set and 100 ng of template DNA according to the manufacturer's instructions and reaction conditions. Reaction products were separated in 25-cm by 25-cm 1.5% agarose gels containing ethidium bromide (3 μg/ml) at room temperature in 1× TBE buffer containing ethidium bromide (1.5 μg/ml) for 14 h at 60 V. Following separation, the DNA bands were visualized by UV transillumination. Rep-PCR results were analyzed using BioNumerics, version 3.5 (Applied-Maths, Kortrijk, Belgium), and banding patterns were compared using Dice coefficients with a 1.0% band position tolerance. Isolate relatedness was determined by using the unweighted-pair group method using average linkages (UPGMA), and a dendrogram was prepared to display their associations.

PFGE. PFGE was performed according to a protocol developed by the Centers for Disease Control and Prevention (CDC) (17). Briefly, agarose-embedded DNA was digested with 50 U of XbaI (Boehringer Mannheim Corporation, Indianapolis, IN) overnight in a water bath at 37°C. The restriction fragments were separated by electrophoresis in 0.5× TBE buffer at 14°C for 18 h, using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA), with pulse times of 2.16 to 63.8 s. The gels were stained with ethidium bromide, and DNA bands were visualized by UV transillumination. PFGE results were analyzed using BioNumerics software, and banding patterns were compared using Dice coefficients with a 1.5% band position tolerance. Isolate relatedness was determined using UPGMA.

MLST. The targets used for MLST included housekeeping genes (*glnA*, *manB*, *pduF*, and the 16S rRNA gene) and factors important in attachment and invasion, including fimbrial genes (*pefB* and *fimH*) and a SPI1 regulator gene (*hilA*) (2, 4, 12, 13). PCR primers were acquired for the glutamine synthetase (*glnA*), phosphomannomutase (*manB*), propanediol utilization factor (*pduF*), and 16S rRNA genes, as described by Kotetishvili et al. (13). Additional primers were designed for a plasmid-encoded fimbria gene (*pefB*), a hyperinvasive locus gene (*hilA*), and a fimbrial gene (*fimH*), using the Vector NTI software package (Oxford Molecular, San Diego, CA) and gene sequences available in GenBank (2, 4, 12). DNA templates were prepared by boiling four or five colonies of each isolate in 200 μl of sterile water, centrifuging the samples, and removing 5 μl of the supernatant fluid for PCR. Each sample was added to a 45-μl reaction mix (50 pmol of each primer, 1.5 mM $MgCl_2$, a 10 mM concentration of each deoxynucleoside triphosphate, and 1.25 units of *Taq* DNA polymerase). PCRs for each of the genes were carried out using AmpliTaq Gold (Perkin-Elmer, Foster City, CA) according to the manufacturer's suggestions, with the following amplification program: 94°C for 10 min; 35 cycles of 94°C for 1 min, 55°C (58°C for *hilA* and *pefB*) for 1 min, and 72°C for 1 min; and 72°C for 5 min. Amplified PCR products were purified using MultiScreen PCR plates (Millipore) according to the manufacturer's instructions.

TABLE 1. PCR and MLST primers used for this study

Gene	Primer sequence (5′→3′)	Reference
glnA	CCGCGACCTTTATGCCAAAACCG	13
	CCTGTGGGATCTCTTTCGCT	13
pduF	CT(C/A)AAAGTCGCYGGYGC	13
	GGGTTCATTGCAAAACC	13
manB	CCGGCACCGAAGAGA	13
	CGCCGCCATCCGGTC	13
16S rRNA	AGTTTGATCATGGCTCAG	13
	TTACCGCGGCTGGCA	13
hilA	TTAATCGTCCGGTCGTAGTG	This study
	TCTGCCAGCGCACAGTAAGG	This study
pefB	TGATGCTGAACAGAAAAGAT	This study
	ATAATAAACAACCATGTGCG	This study
fimH	GGGACGGCGACCGATATCTT	This study
	TTGTCTGGCGAGGGATCGTC	This study

Purified DNAs were subjected to cycle sequencing with the primers listed in Table 1, using fluorescent dye-labeled dideoxynucleotides according to the manufacturer's instructions (ABI Prism BigDye Terminator v3.0 ready reaction sequencing kit; Applied Biosystems, Foster City, CA). The products were separated with 6% denaturing gel polymer under standard conditions in an ABI Prism 3700 automatic sequencer (Applied Biosystems). Sequence results were inspected manually, using the Chromas 2 sequence-viewing program (Technelysium, Helensvale, Australia). Sequence data for each strain were imported into BioNumerics for analysis, and multiple sequence alignments were performed. Sequences were classified into allele types based on sequence variability, and the allele sequence most commonly observed was designated allele 1. The allele types for all gene loci were combined to generate the multilocus sequence type for a particular strain.

Nucleotide sequence accession numbers. Sequences for the allele types generated by MLST were submitted to GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and assigned the accession numbers AY712712 to AY712789.

RESULTS

Antimicrobial susceptibility phenotypes. Thirty of the 128 *S. enterica* serovar Typhimurium isolates were susceptible to all tested antimicrobials, with 43% (13/30) recovered from either chicken diagnostic samples (n = 8/30) or raw chicken meat (n = 5/30). Seventy-seven percent (n = 98/128) of serovar Typhimurium isolates exhibited resistance to at least one antimicrobial, and 70 (55%) of these isolates were resistant to five or more of the tested antimicrobial agents. *Salmonella* serovar Typhimurium isolates displayed resistance most often to sulfamethoxazole (63%), streptomycin (63%), tetracycline (63%), ampicillin (63%), chloramphenicol (46%), kanamycin (37%), and to a lesser extent, cephalothin (27%), amoxicillin-clavulanic acid (25%), ceftiofur (25%), and gentamicin (16%) (Table 2). All isolates were susceptible to amikacin and ciprofloxacin, but 19 (16%) displayed resistance to nalidixic acid and were almost exclusively associated with either turkey diagnostic samples (n = 8/19) or ground turkey isolates (n = 10/19) (Table 2).

Thirty-eight distinct antimicrobial resistance patterns were observed among the 128 *S. enterica* serovar Typhimurium isolates (Fig. 1). The most common resistance pattern was the DT104-associated phenotype, with resistance to Amp, chloramphenicol, Str, Sul, and tetracycline, which was seen among 22 isolates. Sixty-eight percent of these isolates were recovered

TABLE 2. Antimicrobial resistance among *Salmonella enterica* serovar Typhimurium isolates (n = 128) by isolate source

Antimicrobial agent	Resistance breakpoint (μg/ml)[a]	% of isolates with resistance[c]								
		Cattle		Swine		Turkey		Chicken		Total (n = 128)
		Diagnostic (n = 20)	Food (n = 12)	Diagnostic (n = 19)	Food (n = 13)	Diagnostic (n = 16)	Food (n = 16)	Diagnostic (n = 19)	Food (n = 13)	
Ampicillin	≥32	95	50	68	46	88	75	26	46	63
Amoxicillin-clavulanic acid	≥32	20	8	0	0	56	63	26	31	25
Apramycin	≥32	0	0	5	0	0	0	0	0	0.8
Cephalothin	≥32	20	17	0	0	63	63	26	31	27
Ceftiofur	≥8	10	8	0	0	56	63	21	31	25
Ceftriaxone	≥64	15	0	0	0	6	0	0	0	0.8
Chloramphenicol	≥32	70	33	63	39	63	69	5	15	46
Tetracycline	≥16	85	50	84	54	81	75	32	31	63
Amikacin	≥32	0	0	0	0	0	0	0	0	0
Kanamycin	≥64	80	25	16	15	69	63	5	8	37
Gentamicin	≥16	25	0	5	8	31	38	5	8	16
Streptomycin[b]	≥64	90	42	79	69	81	81	21	31	63
Sulfamethoxazole	≥512	95	50	79	69	81	69	26	23	63
Trimethoprim-sulfamethoxazole	≥4	25	8	5	0	0	0	0	0	6
Nalidixic acid	≥32	0	0	0	0	56	63	5	0	16
Ciprofloxacin	≥4	0	0	0	0	0	0	0	0	0

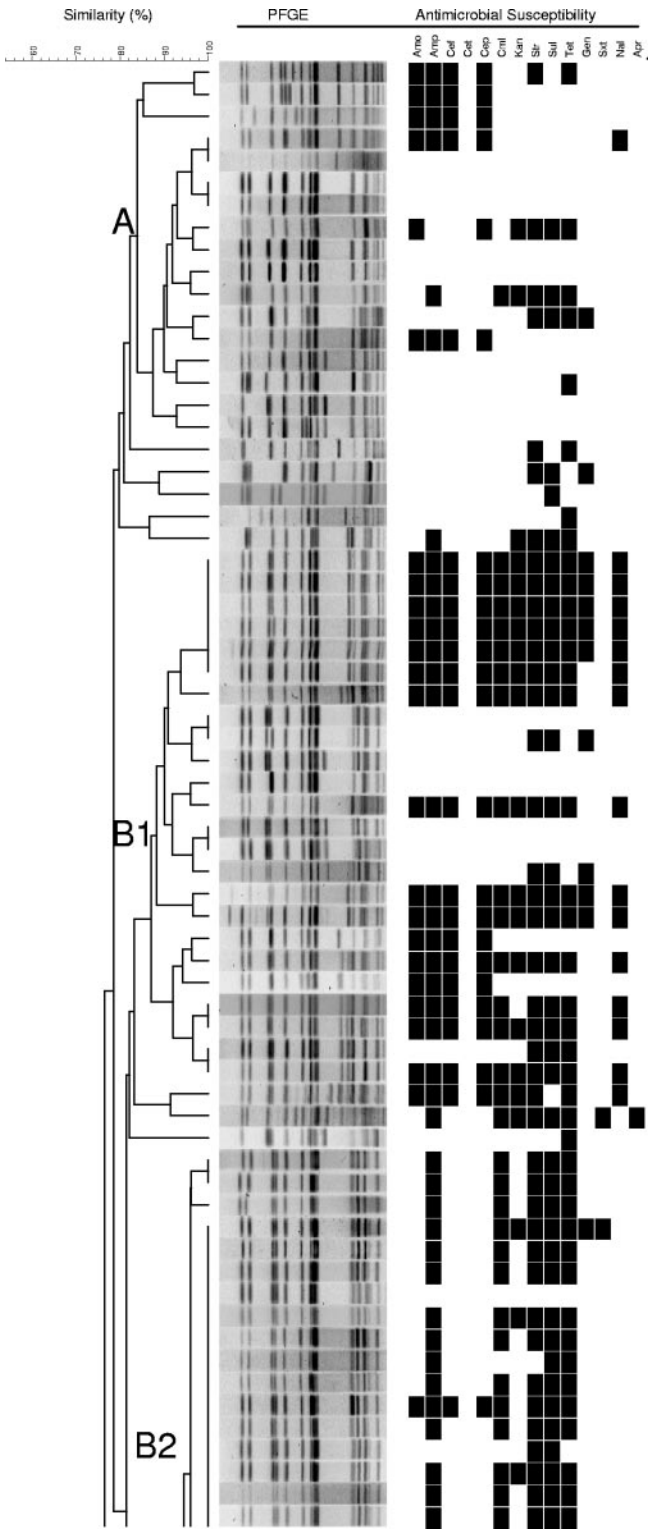
[a] The MIC determined via microdilution broth methods in accordance with CLSI standards (18).
[b] Interpretive criteria have not been established by the CLSI.
[c] Diagnostic, isolates from food animals; food, isolates from a food animal processing or slaughter facility.

from either swine diagnostic samples (n = 10/22) or ground pork samples (n = 5/22). Trimethoprim-sulfamethoxazole resistance was most often observed in serovar Typhimurium isolates recovered from cattle diagnostic samples (25%) or ground beef samples (8%), whereas gentamicin resistance was most often seen in isolates obtained from either turkey diagnostic samples (31%) or ground turkey samples (38%) (Table 2). Resistance to amoxicillin-clavulanic acid and ceftiofur was also most often observed among serovar Typhimurium isolates recovered from either turkey diagnostic samples (56%) or ground turkey samples (63%), while ceftriaxone resistance was identified only among isolates recovered from turkey diagnostic isolates (6%) (Table 2).

Plasmid profiles. Thirty-six different plasmid profiles were generated among the 128 *S. enterica* serovar Typhimurium isolates in this study (Table 3). Forty-seven isolates lacked any type of visible plasmid bands. The sizes of the most commonly identified plasmids were 3.7 kb (n = 30), 2.2 kb (n = 21), and 5.4 kb (n = 19). Twenty-three isolates possessed three or more plasmids of different sizes, and two isolates contained five plasmids. At times, the plasmid contents of the serovar Typhimurium isolates did not completely correlate with the animal source. For example, of the seven serovar Typhimurium isolates that possessed similar plasmid patterns of 5.4, 3.7, and 2.2 kb, five were recovered from ground turkey, one was recovered from a turkey diagnostic sample, and one was recovered from a swine diagnostic sample (Table 3). However, plasmid contents did not show a relationship to antimicrobial resistance phenotypes, Rep-PCR profiles, PFGE patterns, or MLST results. The same seven serovar Typhimurium isolates that possessed similar plasmid patterns of 5.4, 3.7, and 2.2 kb were completely dissimilar with regards to Rep-PCR profiles, PFGE patterns, and MLST results (Fig. 1). Overall, serovar Typhimurium plasmid results were very reproducible using the two different commercial kits, with the exception of two isolates in which a 20-kb plasmid was detected using the Wizard kit but not the Millipore kit.

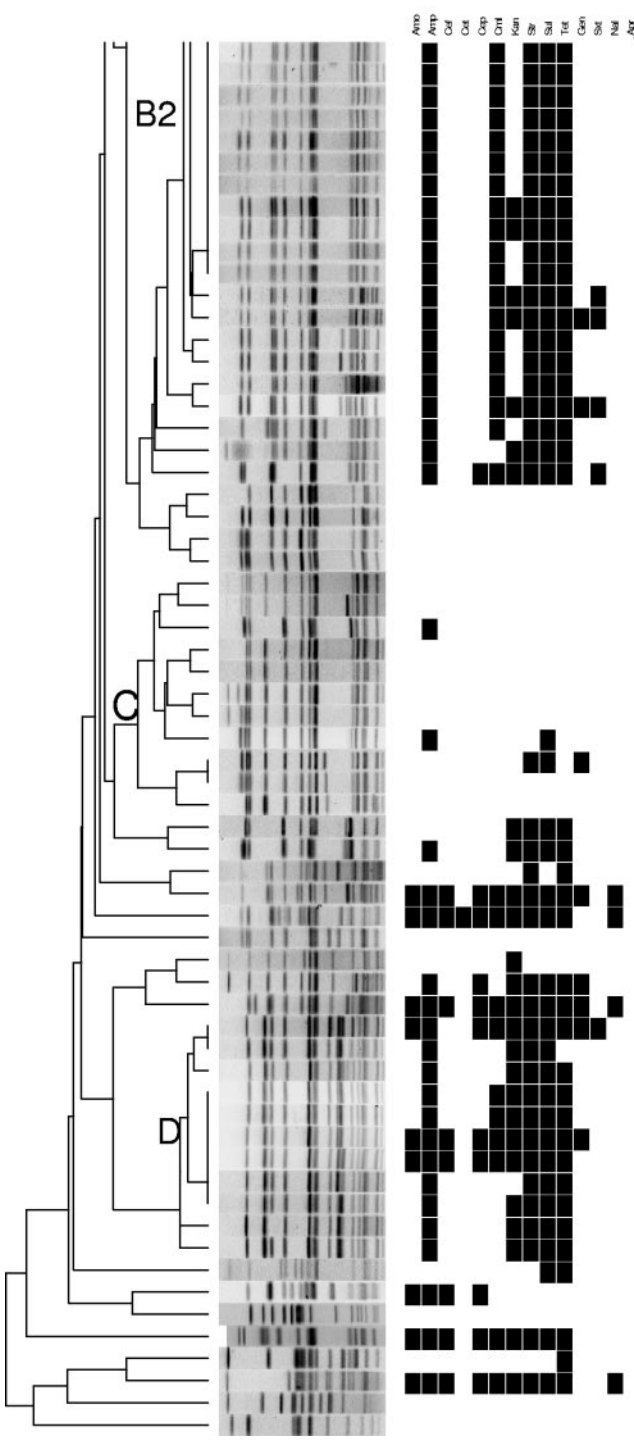
Rep-PCR profiles. Eighty-four different Rep-PCR profiles were obtained among the 128 *S. enterica* serovar Typhimurium isolates by use of a commercially available typing kit (Fig. 1). The two largest groupings of isolates based on Rep-PCR each included five isolates (Rep-PCR patterns 57 and 59). Outside these groupings, the majority of clustering occurred through isolate pairing. There were 20 sets of pairs, four groups contained three isolates, and two groups contained four isolates, with the remainder of the isolates having apparently unique Rep-PCR profiles. There were mixed results among the two most common Rep-PCR profiles compared with the PFGE patterns (Fig. 1). The five serovar Typhimurium isolates designated as having Rep-PCR pattern 57 were all grouped into the B2 PFGE cluster, whereas the Rep-PCR pattern 59 isolates were spread out among the A, B2, and D PFGE clusters (Fig. 1). There was a greater agreement between the next two common Rep-PCR patterns and the PFGE results, as three of four isolates comprising Rep-PCR pattern 24 and two of four isolates included in Rep-PCR pattern 30 were indistinguishable by PFGE. However, discordant results were also observed, as 18 distinct Rep-PCR patterns were identified among the 25 serovar Typhimurium isolates comprising the largest indistinguishable PFGE pattern in cluster B2 (Fig. 1).

PFGE patterns. A total of 86 distinct PFGE patterns were generated among the 128 *S. enterica* serovar Typhimurium isolates in this study (Fig. 1). Four large clusters (A to D) containing at least 10 different PFGE patterns were defined, using an 80% similarity index (Fig. 1). Cluster B was the largest and included 65 isolates which were further separated into two distinct subclusters (B1 and B2), each with a >85% similarity. The largest indistinguishable PFGE pattern contained 25 isolates and was located in cluster B2. *Salmonella* serovar Typhimurium isolates comprising this pattern were recovered pri-



CVM #	Animal Source	Plasmids Present (Kb)	Rep-PCR	MLST Alleles g	pd	m	16	pe	h	f	Sequence Type
7473	Chicken		52	1	12	1	1	1	2	1	69
7529	Chicken	10.6	43	1	1	9	1	1	2	1	35
4623	Chicken	4.5, 3.9, 2.2	59	1	1	1	1	1	4	1	8
4628	Chicken	20.0, 5.0, 3.2, 2.8, 2.2	59	1	3	1	1	1	16	1	46
7494	Chicken	3.8, 2.3	39	1	3	1	1	1	2	1	44
4545	Ground pork		29	1	1	4	2	2	4	1	32
4624	Chicken	20.0	59	1	1	4	1	1	9	1	31
4270	Chicken		61	1	3	1	1	1	1	1	43
7559	Ground beef	5.4, 2.2	45	1	1	4	1	1	2	1	30
4486	Chicken carcass		28	1	17	1	1	4	2	1	81
4532	Ground chicken	6.4, 3.8	67	1	1	1	1	3	4	1	14
4507	Chicken carcass	5.4, 2.2	66	1	1	1	7	1	2	1	27
5829	Chicken carcass	2.2	68	1	1	1	1	1	1	3	3
5861	Ground pork	7.0	73	1	15	1	1	1	2	1	75
7521	Chicken	7.3, 2.2	38	1	1	1	1	1	1	1	1
7426	Ground pork		82	1	9	1	9	1	2	1	61
7450	Ground beef		84	1	13	1	2	1	2	1	72
4482	Chicken carcass		66	1	12	1	1	1	13	1	70
4520	Ground turkey	6.1, 4.9	19	1	1	1	2	1	1	1	16
7503	Chicken	20.0	42	1	15	1	1	1	2	1	75
7492	Chicken	2.2	50	1	1	1	2	1	2	1	17
7549	Ground pork	4.3, 4.0	44	1	1	1	1	1	1	1	1
7542	Ground turkey	5.8, 3.7	31	1	5	1	1	1	3	1	51
7545	Ground turkey	5.4, 3.7, 2.2	31	1	1	1	1	1	1	1	1
7551	Ground turkey	5.4	53	1	1	1	1	1	1	1	1
7866	Turkey	5.1, 3.7	14	1	9	1	8	1	1	1	60
7869	Turkey	5.8, 3.7	33	1	1	1	8	1	3	1	29
7870	Turkey	5.4	11	1	1	1	1	1	1	1	1
7490	Ground turkey	5.4, 3.7, 2.2	36	1	16	1	1	1	2	1	79
4540	Ground pork	10.6	26	1	5	1	2	1	1	4	52
7773	Swine		8	1	15	112		1	1	1	78
4598	Ground beef		71	1	14	1	3	1	1	4	73
4506	Ground turkey	6.6, 6.1, 5.1	55	1	1	1	1	1	1	1	1
7864	Turkey	5.4, 3.7, 2.2	4	1	1	1	1	1	2	5	5
4460	Ground beef	6.1, 5.6, 3.7	9	1	1	1	5	1	23	1	25
7940	Cattle		60	1	1	1	2	1	3	1	18
7479	Chicken		48	1	1	1	2	1	1	1	16
7512	Ground turkey	5.4, 3.7	41	4	3	1	1	1	2	1	89
7868	Turkey	5.4	32	1	15	1	1	1	1	1	74
4574	Chicken carcass	2.0	10	1	1	1	1	1	2	1	4
7860	Turkey	5.4	75	1	1	148		1	1	1	39
4601	Chicken carcass	2.2	80	1	1	1	1	1	12	1	11
7505	Ground turkey	3.7	68	1	1	1	1	1	6	1	9
7556	Ground turkey	5.4, 3.7, 2.2	10	1	4	101		1	3	1	50
4473	Ground pork	7.3, 6.1, 3.7	15	1	17	1	1	1	6	1	80
7523	Ground turkey	5.1, 3.7	37	1	3	1	1	1	2	1	44
7537	Ground turkey	5.4, 3.7, 2.2	43	1	1	1	1	1	11	1	10
7764	Swine	20.0, 6.1, 3.7	65	1	1	1	1	1	3	2	7
4608	Swine		79	1	15	1	7	1	2	1	76
4463	Ground turkey		5	1	3	1	1	1	2	1	44
799	Swine	3.7	18	1	2	1	2	1	1	1	41
797	Swine	3.7	58	1	1	1	2	1	1	1	16
1020	Cattle	5.4	57	1	1	1	1	1	2	1	4
4484	Chicken carcass		20	4	1	1	1	1	3	1	87
4517	Ground pork	3.7	12	1	3	1	1	1	15	1	45
4522	Ground beef	11.3, 5.8	7	1	1	1	1	1	1	1	1
4533	Ground beef	5.8, 3.7	16	1	1	1	1	1	2	1	4
5840	Ground pork	11.3, 8.9, 5.1, 3.4, 1.4	69	1	1	1	1	1	2	1	4
5851	Ground beef		70	1	1	1	6	1	2	1	26
5858	Ground pork	3.4	78	1	1	1	2	1	1	1	16
7425	Ground beef	6.1, 3.5, 2.5	78	1	1	1	1	1	1	1	1
7431	Cattle	2.4	40	1	9	1	1	1	1	1	57
7442	Ground pork	5.8, 3.7	83	1	9	1	2	1	3	1	59
7445	Ground pork	6.4	24	1	1	1	1	1	1	1	1
7482	Chicken	4.9	51	1	1	1	1	1	2	1	4
7516	Ground pork	2.2	34	2	1	1	1	1	1	1	82

FIG. 1. UPGMA analysis of PFGE profiles of *S. enterica* serovar Typhimurium isolates, showing PFGE fingerprints (80% similarity), animal sources, antibiotic susceptibility patterns (a black box indicates resistance to the particular antimicrobial agent; all isolates were susceptible to amikacin and ciprofloxacin and thus were not included in the figure), Rep-PCR profiles, plasmid profiles, and sequence types (ST). Major clusters are marked A, B1, B2, C, and D. Columns g (*glnA*), pd (*pduF*), m (*manB*), 16 (16S rRNA gene), pe (*pefB*), h (*hilA*), and f (*fimH*) provide the individual sequence allele types that were combined to generate the MLST. The number for each allele corresponds to the sequence alleles listed in the GenBank database under accession numbers AY712712 to AY712789. The dendrogram has been split to facilitate text legibility, with the top half on the left and the continuation on the right.



CVM #	Animal Source	Plasmids Present (Kb)	Rep-PCR	MLST Alleles g pd m 16 pe h f	Sequence Type
7516	Ground pork	2.2	34	2 1 1 1 1 1 1	82
7566	Ground beef	20.0	12	1 1 1 1 1 2 1	4
785	Swine	6.1, 5.1, 3.7	30	1 1 1 1 1 1 1	1
7859	Turkey		24	4 3 1 1 1 1 1	88
786	Swine	6.1, 5.1, 3.7	26	1 1 1 2 1 4 1	19
787	Swine	6.1, 5.1, 3.7	27	1 1 1 2 1 7 1	22
788	Swine	6.1, 5.1, 3.7	27	2 12 4 1 1 1 1	83
7912	Cattle	6.1, 3.7, 2.2	24	1 1 1 1 4 14 1	15
7928	Cattle	3.7	13	1 1 1 1 1 2 1	4
796	Swine		59	1 1 1 1 1 3 1	6
798	Swine		57	1 3 1 2 1 1 1	47
1017	Cattle	20.0, 6.1, 3.7	57	1 1 1 1 1 2 1	4
1018	Cattle	20.0, 6.1, 3.7	57	1 1 1 1 1 1 1	1
4583	Swine		80	1 1 1 1 1 1 1	1
4591	Swine	5.4, 3.7, 2.2	81	1 1 1 1 1 2 1	4
1021	Cattle	5.4	57	1 1 1 2 1 1 1	16
1029	Cattle		20	1 1 1 8 1 3 1	29
4521	Swine	6.3, 3.1	12	1 1 1 2 1 10 1	23
7934	Cattle	2.4	13	1 1 121 1 2 1	36
4578	Ground beef		82	1 1 1 1 1 1 1	1
5870	Ground beef	5.4	64	1 15 1 7 1 2 3	77
7447	Chicken		21	1 2 1 1 1 1 1	40
4518	Chicken		56	1 4 1 1 1 3 1	49
7567	Chicken		46	1 1 1 1 1 2 1	4
4271	Chicken	7.0	6	1 9 7 2 1 9 1	64
4314	Turkey		55	1 1 1 2 1 1 1	16
7526	Ground turkey	4.9	26	1 1 1 1 3 1 1	13
4656	Chicken		18	1 1 132 1 18 1	37
7454	Chicken	6.1, 4.9	50	1 9 1 2 1 2 1	58
4543	Ground chicken		66	1 11 1 2 1 1 1	67
4582	Chicken carcass		72	1 1 1 2 1 2 1	17
1031	Cattle		30	1 1 1 1 1 4 1	8
4498	Ground pork		30	1 6 1 2 1 3 1	53
4523	Ground chicken		30	1 11 6 2 1 17 1	68
4563	Ground chicken		24	1 10 1 2 1 2 1	66
4485	Swine		31	1 12 1 1 1 2 1	69
7789	Swine		1	1 3 3 1 1 1 1	48
5828	Ground turkey	4.5, 2.2	79	1 1 1 1 1 2 1	4
7475	Ground turkey	5.4, 3.7, 2.2	35	1 9 8 2 1 1 1	65
7863	Turkey	5.4	76	2 14 1 2 1 1 1	84
4478	Turkey	9.9, 6.1	77	1 8 1 2 1 2 1	56
7477	Ground turkey		70	1 1 1 1 1 1 1	1
7865	Turkey	9.9, 5.8, 3.7	33	1 1 1 1 1 1 1	1
7861	Turkey		21	1 1 1 1 1 1 2	2
7913	Cattle		60	1 8 1 1 1 1 1	55
7950	Cattle	6.4	54	1 1 1 2 1 2 1	17
7517	Ground beef		49	1 9 2 2 1 2 1	62
1904	Cattle		22	1 1 1 7 1 7 1	28
1905	Cattle		23	1 1 1 2 1 5 1	20
2985	Cattle		29	1 1 5 2 1 5 1	33
2989	Cattle		25	1 9 5 2 1 2 1	63
7874	Turkey		59	1 2 5 2 1 3 1	42
7955	Cattle		34	1 1 1 2 1 6 1	21
7872	Turkey	2.4	62	1 1 5 2 1 5 1	33
7927	Cattle		8	1 1 5 4 1 2 1	34
7463	Chicken	7.0	47	1 1 1 2 2 2 1	24
4607	Chicken carcass	2.2	64	1 1 1 1 1 1 1	1
7796	Swine	2.4	3	1 7 1 3 1 19 2	54
1028	Cattle	3.4	23	1 1 1 1 1 20 1	12
7811	Swine		17	3 7 143 1 8 1	85
7862	Turkey	4.5, 2.2	2	3 7 143 1 21 6	86
7873	Turkey	2.4	63	1 12 143 1 22 5	71
7813	Swine		74	1 1 142 1 8 1	38

FIG. 1—*Continued.*

marily from swine diagnostic samples and derived food products ($n = 6$ for swine; $n = 7$ for ground pork) or cattle diagnostic samples and derived food products ($n = 4$ for cattle; $n = 5$ for ground beef). Additionally, the majority of these isolates (16/25) exhibited resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline, which is the typically observed DT104 pentaresistance pattern. The majority of the remaining isolates had unique PFGE profiles, with the exception of two indistinguishable patterns containing six isolates each and nine pairs with shared profiles. The two patterns containing six isolates each were grouped into either cluster B1 or D and correlated well with food animal origins, as 100% of isolates with one pattern consisted of serovar Typhimurium isolates recovered from either turkey

TABLE 3. *Salmonella enterica* serovar Typhimurium plasmid profiles by isolate source

Plasmid size(s) (kb)	No. of isolates with plasmid(s)[a]								Total (n = 128)
	Cattle		Swine		Turkey		Chicken		
	Diagnostic (n = 20)	Food (n = 12)	Diagnostic (n = 19)	Food (n = 13)	Diagnostic (n = 16)	Food (n = 16)	Diagnostic (n = 19)	Food (n = 13)	
2.0								1	1
2.2				1			1	3	5
2.4	1	1	1		2				5
3.4	1			1					2
3.7	1		2	1		1			5
4.9						1	1		2
5.4	2	1			4	1			8
6.4	1			1					2
7.0				1			2		3
10.6				1			1		2
20.0		1					2		3
11.3, 5.8		1							1
11.3, 8.9, 5.1, 3.4, 1.4				1					1
20.0, 5.0, 3.2, 2.8, 2.2							1		1
20.0, 6.1, 3.7	2		1						3
3.8, 2.3							1		1
4.3, 4.0				1					1
4.5, 2.2					1	1			2
4.5, 3.9, 2.2							1		1
5.1, 3.7					1	2			3
5.4, 2.2		1						1	2
5.4, 3.7, 2.2			1		1	5			7
5.8, 3.7		1		1	1	1			4
6.1, 3.5, 2.5		1							1
6.1, 3.7, 2.3	1								1
6.1, 4.9						1	1		2
6.1, 5.1, 3.7			4						4
6.1, 5.6, 3.7		1							1
6.3, 3.1			1						1
6.4, 3.8								1	1
6.6, 6.1, 5.1						1			1
7.3, 2.2							1		1
7.3, 6.1, 3.7				1					1
9.9, 5.8, 3.7					1				1
9.9, 6.1					1				1
None Visible	11	4	9	3	4	2	7	7	47

[a] Diagnostic, isolates from food animals; food, isolates from a food animal processing or slaughter facility.

diagnostic samples or ground turkey and five of six isolates with the other pattern were isolated from cattle diagnostic samples.

With regard to specific PFGE patterns associated with *S. enterica* serovar Typhimurium isolates recovered from different animals and animal-derived products, 19 patterns were generated from 32 cattle/ground beef isolates, 19 patterns were generated from 32 swine/ground pork isolates, 26 patterns were generated from turkey/ground turkey isolates, and 30 patterns were generated from chicken/raw chicken isolates.

MLST profiles. In order to compare single nucleotide polymorphisms against the whole genome profile provided by PFGE, MLST was conducted on all 128 isolates, comparing partial DNA sequences of seven genes (*glnA*, *manB*, *pduF*, the 16S rRNA gene, *pefB*, *fimH*, and *hilA*) (Table 1). Between 4 and 23 alleles per gene were identified among the 128 serovar Typhimurium isolates (Table 4). Four allele types were found among *glnA* sequences, with 118 of the isolates sharing a common sequence. Representative sequences for each of the allele types are available in the GenBank database under accession numbers AY712727 to AY712730 and represent allele types 1 to 4 (with allele type 1 being the wild type). Seventeen distinct allele types were found among *pduF* sequences (accession numbers AY712769 to AY712785), 14 allele types were found among *manB* sequences (accession numbers AY712755 to AY712768), 9 allele types were found for 16S rRNA gene sequences (accession numbers AY712712 to AY712720), 4 allele types were found for *pefB* sequences (accession numbers AY712786 to AY712789), 23 allele types were found for *hilA* sequences (accession numbers AY712732 to AY712754), and 6 allele types were found for *fimH* sequences (accession numbers AY712721 to AY712726). The results of the individual gene sequences were combined to generate 89 distinct multilocus sequence types (Fig. 1). The most commonly detected sequence type (ST 1), which had the allele profile 1-1-1-1-1-1-1 for *glnA*-*pduF*-*manB*-16S rRNA-*pefB*-*hilA*-*fimH*, was found in 16 (12.7%) of the 128 serovar Typhimurium isolates screened. The other most frequent MLST patterns were 1-1-1-1-1-2-1 (ST 4) and 1-1-1-2-1-1-1 (ST 16), which were detected in 11 (8.6%) and 6 (4.7%) of the isolates. Two other sequence types were generated by three isolates each, and five sequence types were common to two isolates.

Although similar numbers of patterns were obtained for the

TABLE 4. MLST alleles of *S. enterica* serovar Typhimurium isolates

Allele[a]	GenBank sequence accession no. or sequence difference from wild type (no. of bases analyzed)						
	glnA (310)	*pduF* (390)	*manB* (588)	16S rRNA (351)	*pefB* (214)	*hilA* (355)	*fimH* (305)
1[b]	AY712727	AY712769	AY712755	AY712712	AY712786	AY712732	AY712721
2	217C→A	175G→T, 220G→A	526T→C, 530G→T	180A→T	168C→C	201T→G	59G→A, 100A→G, 104T→C
3	227G→A	175G→T	522A→C, 530G→T	23C→T, 180A→T	32G→C	201T→G, 230T→C, 287A→T, 337A→G	4C→T
4	249C→T	113T→C, 175G→T	562C→T	180A→T, 223G→C	159T→C	201T→G, 230T→C, 287A→T	72C→T
5		257T→G	440G→A	180A→T, 117G→T		287A→T	104T→C
6		257T→C	525G→A	180A→T, 197C→T		201T→G, 239A→T	104T→C, 164C→A, 211T→G, 226A→C, 244A→T
7		352C→T	298C→T	27G→C		201T→G, 287A→T	
8		190G→C	139T→A	27G→T, 28A→C		37C→T, 138T→A, 195C→T, 222T→C, 345A→G	
9		76T→G	388C→G	27G→T		201T→G, 230T→C, 287A→T, 318G→C	
10		220G→A	176C→A			201T→G, 230T→C, 238A→T	
11		175G→T, 267G→A	545A→C			201T→G, 231T→C, 239A→T	
12		267G→A	543G→C			201T→G, 275T→G	
13		247G→T, 267G→A	132C→T, 374C→A			20A→T	
14		25C→G	128C→T, 146G→C, 208G→C, 212T→C, 249T→G, 251T→G, 254C→T, 255G→T, 263T→C, 302C→T			230T→C, 287A→T	
15		25C→G, 175G→T	440G→A, 495A→C, 525G→C, 528G→C			201T→G, 318G→C	
16		25C→G, 175G→T, 248T→A				201T→G, 287A→T, 318G→C	
17		168T→A, 247G→T				25G→T, 201T→G, 287A→T	
18						123C→G, 201T→G, 287A→T	
19						96G→C, 138T→A, 201T→G	
20						201T→G, 230T→C	
21						138T→A, 195C→T, 222T→C, 345A→G	
22						97C→A, 120C→T, 132G→A, 222T→C, 345A→G	
23						201T→G, 204T→G, 231T→C, 287A→T	

[a] Alleles found for each of the genes sequenced; allele differences are listed by base changes at the indicated positions from the wild-type sequence.
[b] Allele 1 is the wild-type sequence for each gene.

128 serovar Typhimurium isolates (86 PFGE versus 89 MLST patterns), MLST appeared to be more discriminatory than PFGE in several instances. For example, the largest indistinguishable PFGE pattern of *S. enterica* serovar Typhimurium isolates ($n = 25$) contained 16 different sequence types (Fig. 1). The next two largest PFGE patterns were also further differentiated by MLST, with four sequence types among six isolates in one case and six sequence types among six isolates in the other (Fig. 1). However, there were also examples of PFGE being more discriminatory than MLST, as 11 of 16 isolates comprising ST 1 were grouped into different PFGE patterns and clusters.

DISCUSSION

One hundred twenty-eight *S. enterica* serovar Typhimurium isolates from bovine, porcine, and avian sources or derived food products were characterized by antimicrobial susceptibility profiling, plasmid profiling, Rep-PCR, PFGE, and MLST. The results of this study indicate that the molecular typing methods have similar abilities to genetically type isolates. Eighty-six different PFGE profiles, 84 Rep-PCR patterns, and 89 multilocus sequence types were identified among the 128 isolates tested. There was less discriminatory power in plasmid and antimicrobial susceptibility profiling, as only 36 different plasmid profiles and 38 different antimicrobial susceptibility profiles were observed among the isolates. These data confirm those of Fernandez et al., who concluded that plasmid typing did not provide enough discriminatory power due to its instability and the low level of diversity of extrachromosomal DNAs harbored among *Salmonella* isolates (9).

When the results of MLST, Rep-PCR, and PFGE were compared to one another, there did not appear to be any correlation of clustered isolates for the different methods. For example, 25 serovar Typhimurium isolates share indistinguishable PFGE patterns in cluster B2 (Fig. 1). However, within this homogenous grouping exist 16 unique MLST patterns and 18 unique Rep-PCR patterns. In some instances, there did appear

to be a relationship between certain PFGE patterns and antimicrobial resistance profiles. Twenty-two of 25 isolates that shared indistinguishable PFGE patterns within cluster B2 exhibited resistance to at least five antimicrobials, including ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline. This PFGE pattern is similar to one we previously associated with serovar Typhimurium definitive type 104 (DT104) isolates displaying similar resistance profiles (29). This trend was also seen for isolates with similar PFGE profiles to that of the homogenous group in cluster B2. All isolates with PFGE patterns that were at least 90% similar to this indistinguishable PFGE pattern also displayed resistance to the same five antimicrobial agents. This suggests that a predominant pulsotype may be circulating among food animals; however, this should be verified with a larger collection of isolates.

The differences in results among the typing methods are likely dependent on the way that genetic diversity is measured. PFGE relies on whole-genome restriction and separation to distinguish isolates. This is a potential improvement over some of the PCR-based typing techniques that rely on amplification of DNAs from a limited number of sequence targets to develop a genetic profile (16, 23). While PFGE is considered the "gold standard" molecular typing method (19), if genetic variation does not significantly impact the size or electrophoretic mobility of a restriction fragment, then the change may not be identified as a separate pulsotype. This limitation can be mitigated to some extent by the use of a second enzyme (for example, BlnI) for PFGE analysis, which has been shown to further increase the discriminatory power for differentiating several bacterial pathogens, including *Salmonella* and *E. coli* O157:H7 isolates (7, 9).

MLST utilizes variability in the sequences of particular genes, due to mutation or recombination events, to determine the relatedness of bacteria. With MLST, multiple genes with conserved sequences are compared for nucleotide base changes (14, 22). Housekeeping genes (genes required for basic cellular functions) are most often sequenced because they are present in all isolates and are not subject to strong selective pressures that can lead to relatively rapid sequence changes. In the present study, insufficient diversity was generated by solely using the targeted housekeeping genes (*glnA*, *manB*, *pduF*, and the 16S rRNA gene). The lack of adequate discrimination among housekeeping genes was in agreement with the results recently reported by Fakhr et al. when they examined serovar Typhimurium isolates that originated in cattle (8). Therefore, we attempted to increase the observed genetic diversity of serovar Typhimurium by adding three nonhousekeeping genes. The additional genes we chose for MLST have been reported to be important in *Salmonella* virulence through attachment to and invasion of host epithelium (2, 3, 27, 29). It was reasoned that there could potentially be some degree of genetic diversity among these *Salmonella* genes with regard to their animal origins. While MLST results using these additional genes did not demonstrate host species specificity, they did detect more diversity, leading to better separation among the serovar Typhimurium isolates to the point that the technique was able to distinguish among isolates of the large, apparently homogenous PFGE grouping in cluster B2, revealing 16 distinct patterns (Fig. 1). Therefore, our results indicate that MLST may be a good molecular epidemiological option for discriminating among isolates that are shown to be genetically indistinguishable by PFGE. This finding is also in agreement with an earlier study which showed that MLST was more discriminatory than PFGE for typing *Salmonella* isolates (13).

An added aim of this study was to evaluate the different typing methods for the ability to discriminate among serovar Typhimurium isolates based upon their food animal sources. As indicated in Fig. 1, the majority of the methods tested did not generate any animal origin-specific clustering. There were, however, a few exceptions to this general trend, as a few resistance patterns were detected that were only seen for isolates from a single food animal species. For example, a number of the turkey isolates (including both diagnostic isolates and those from ground product) had unique resistance profiles for 10 or more antimicrobial agents. Many of the same isolates possessed similar PFGE profiles as well. A second grouping was seen which contained seven cattle diagnostic and ground beef isolates, which possessed very similar PFGE profiles and displayed resistance to at least Amp, kanamycin, Str, and Sul. If these PFGE and antimicrobial resistance patterns remain exclusive to turkey or cattle isolates in a larger setting, then the detection of these profiles in a strain isolated from a human with salmonellosis may serve as a tool to indicate the potential source of the pathogen.

We evaluated a number of typing methods to distinguish among serovar Typhimurium isolates of food animal origin and found that both PFGE and MLST provide good discriminatory power to differentiate isolates. Rep-PCR appeared to work well to genetically type isolates, but the technique had problems with analysis and reproducibility, making it difficult to make relational inferences. We do look forward to continued efforts to automate Rep-PCR and increase its performance and reproducibility, as this technique holds much promise. Antibiotic resistance profiling and plasmid profiling will typically not provide adequate discrimination among isolates compared to the molecular typing methods, with a few noted exceptions. Collectively, the results demonstrate that the use of a combination of methods increases the ability to discriminate among strains, especially when trying to separate isolates that appear clonal by a single technique. This finding agrees with the recent results of Tatavarthy et al., who examined the genetic diversity of *S. enterica* serovar Newport isolates (25). The combinational approach has promise to be especially valuable in many public health settings, where the determination of the origin of a pathogen is required for intervention. Multiple methods could help to eliminate suspected sources from the search for the pathogen's origin, leading to increased specificity in health monitoring.

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